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RESEARCH ARTICLE

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Oxidative stress induces *MUC5AC* expression through mitochondrial damage-dependent STING signaling in human bronchial epithelial cells

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Abstract

Oxidative stress increases the production of the predominant mucin MUC5AC in airway epithelial cells and is implicated in the pathogenesis of bronchial asthma and chronic obstructive pulmonary disease. Oxidative stress impairs mitochondria, releasing mitochondrial DNA into the cytoplasm and inducing inflammation through the intracytoplasmic DNA sensor STING (stimulator of interferon genes). However, the role of innate immunity in mucin production remains unknown. We aimed to elucidate the role of innate immunity in mucin production in airway epithelial cells under oxidative stress. Human airway epithelial cell line (NCI-H292) and normal human bronchial epithelial cells were used to confirm *MUC5AC* expression levels by real-time PCR when stimulated with hydrogen peroxide (H_2O_2). *MUC5AC* transcriptional activity was increased and mitochondrial DNA was released into the cytosol by H_2O_2. Mitochondrial antioxidants were used to confirm the effects of mitochondrial oxidative stress where antioxidants inhibited the increase in *MUC5AC* transcriptional activity. Cyclic GMP-AMP synthase (cGAS) or STING knockout (KO) cells were generated to investigate their involvement. H_2O_2-induced *MUC5AC* expression was suppressed in STING KO cells, but not in cGAS KO cells. The epidermal growth factor receptor was comparably expressed in STING KO and wild-type cells. Thus, mitochondria and STING play important roles in mucin production in response to oxidative stress in airway epithelial cells.

KEYWORDS

hydrogen peroxide, mitochondria, mtDNA, MUC5AC, oxidative stress, STING

1 | INTRODUCTION

Airway mucins play a role in preventing water loss and controlling the body's defense against inhaled foreign substances, such as microbes, inflammatory cells, and pollutant particles.[1] However, overproduction of mucins aggravates airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD). In severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infection, the excessive production of airway mucin is associated with disease severity.[2] Therefore, mucin production must be controlled to maintain lung homeostasis. The predominant mucin in the airway is MUC5AC, which is primarily secreted by goblet cells in the airways. *MUC5AC* expression is increased by a wide variety of factors, such as environmental particles, inflammatory cytokines, viral components, several growth factors,[3] and oxidative stress.[4]

Oxidative stress causes mitochondrial damage,[5,6] followed by the release of mitochondrial DNA (mtDNA) into the cytoplasm.[5] Cytoplasmic DNA activates innate immunity through several types of DNA sensor systems, including toll-like receptors, nucleotide-binding oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 inflammasome, and the cyclic GMP-AMP (cGAMP) synthase (cGAS)-stimulator of interferon genes (STING) pathway. STING has been implicated in the activation of antiviral and antitumor immunity and autoimmune disease onset.[7] STING is known to play a key role in the cellular response to cytoplasmic mtDNA.[8,9]

STING plays a role in lung disease,[10] where the cGAS-STING pathway is involved in lung inflammation and fibrosis.[11] However, its involvement in mucin production in airway epithelial cells has not been elucidated. Furthermore, the relationship between oxidative stress-induced mitochondrial damage and airway mucin production has not yet been reported.

Therefore, we hypothesized that oxidative stress-induced transcription of *MUC5AC* may be dependent on mitochondrial damage leading to the release of mtDNA into the cytoplasm and thus the activation of STING DNA sensing pathway. We tested this hypothesis using normal human bronchial epithelial (NHBE) and NCI-H292 cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Normal human bronchial epithelial cells (Lonza, Lot 20TL119095, Lot 0000328324) were cultured at an air–liquid interface (ALI) according to a previously reported protocol, with minor modifications.[12] Cells were maintained in plastic T-75 flasks (Corning, 430641U) and grown in a bronchial epithelial growth medium (BEGM Bullet Kit: Lonza CC-3170) supplemented with bovine pituitary extract, hydrocortisone (0.5 μg/mL), recombinant human epidermal growth factor (EGF) (0.5 ng/mL), epinephrine (0.5 μg/mL), transferrin (10 μg/mL), insulin (5 μg/mL), retinoic acid (0.1 ng/mL), triiodothyronine (6.5 ng/mL), gentamycin sulfate (50 μg/mL), and amphotericin B (50 ng/mL) at 37°C and 5% CO_2 in a humidified incubator. The medium was changed every 48 h until the cells reached 80%–90% confluency. Cells were then seeded at 3.0×10^5 cells/mL into transwell-clear culture inserts (6.5-mm, 0.4-μm pore size Corning, Costar 3470). A differentiation medium containing a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Gibco 12320032, Thermo Fisher Scientific) and BEGM supplemented as mentioned previously excluding triiodothyronine and retinoic acid and including 50 nM all-trans retinoic acid (Sigma-Aldrich, R2625) was used. Cells were submerged in the medium for 7 days after which the apical medium was removed, and ALI was maintained for the remainder of the culture period until 2 days before stimulation. The medium was replaced thrice per week. The apical surface of the cells was rinsed with PBS on day seven of ALI induction. Stimulation and assays were performed on days 11–14 after initiation of ALI.

The human pulmonary mucoepidermoid carcinoma cell line (NCI-H292; ATCC CRL-1848) was maintained in Roswell Park Memorial Institute (RPMI-1640) medium (Gibco 22400089, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C in a humidified atmosphere with 5% (v/v) CO_2. NCI-H292 cells were seeded into 24-well plates (Nunc 142475, Thermo Fisher Scientific) for mRNA analysis, 6-well plates (Falcon 353046, Corning) for Western blotting and DNA extraction, and 6-cm dishes (Falcon 353002, Corning) for DNA extraction. Cells were grown for about 80%–90% confluence and were maintained overnight in serum-free medium before stimulation.

2.2 | Stimulation of cells

For oxidative stress, hydrogen peroxide (H_2O_2) solution (30%) (Fujifilm Wako, 084-07441) was diluted 1000-fold with the culture medium and added to both the apex and bottom of the ALI. Cells were preincubated for 1 h with Mito-TEMPO (Hydorate, Cayman Chemical 16621) or 2 h with the STING inhibitor, H-151 (InvivoGen inh-h151) and then stimulated with H_2O_2.

For intracellular DNA provocation, NCI-H292 cells were transfected with 0.5 μg of poly(dA:dT) naked (Invivogen, tlrl-patn) using the FuGENE HD Transfection Reagent (Promega, E2311) and incubated for 24 h. 2′3′ cGAMP (InvivoGen, tlrl-cga23-s) was dissolved in double-distilled water at 1 mg/mL and stored at −20°C until use. Cells were stimulated with 2′3′ cGAMP for 16 h. MitoTEMPO (Cayman Chemical, 16621) was preadministered to cells 1 h before H_2O_2 stimulation. Thapsigargin (Cayman Chemical, 10522) was dissolved in dimethyl sulfoxide at 1 mM and stored at −20°C until use. Thapsigargin, which can trigger mitochondrial DNA release into the cytosol,[13] was diluted with culture medium to appropriate concentrations, incubated for 4 h, and further cultured in serum-free RPMI-1640 for 20 h. Cells were incubated with a medium containing recombinant human transforming growth factor-alpha (TGF-α; R&D 239-A-100), an EGF receptor (EGFR) agonist for 6 h.

2.3 | Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was prepared using a previously described method[14,15] with a slight modification from Kentucky 1R6F research-reference filtered cigarettes purchased from the University of Kentucky Cigarette Research and Development Center (Lexington, KY, USA). CSE was freshly prepared by mixing smoke from eight cigarette bubbles in 60 mL of PBS. Each cigarette was smoked for 3–4 min, leaving a 20-mm butt. This solution was considered as 100% CSE, adjusted to pH 7.4, and filtered through a 0.22-μm filter prior to cell treatment. CSE was diluted to an appropriate concentration in a serum-free medium before stimulation and was added to the culture medium.

2.4 | Cell viability assay

The cell viability assay was performed as previously described.[16] Briefly, cells were grown in a 35-mm dish, harvested by trypsinization, and resuspended in 2 mL of RPMI 1640 medium with FBS. The cells were enumerated using a cytometer (Countess automated cell counter, Invitrogen, C10281, Thermo Fisher Scientific), and the number of viable cells was determined based on 0.4% Trypan Blue dye exclusion.

2.5 | Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was isolated using the TRIzol reagent (Thermo Fisher Scientific, 15596-018). Reverse transcription was performed with 500 ng of total RNA and oligo (dT) primers using SuperScript III (Invitrogen, 10308632), according to the manufacturer's protocol. Relative mRNA levels were quantified using the TaqMan® Gene Expression Master Mix (Applied Biosystems, 4369016, Thermo Fisher Scientific) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Threshold cycle time (CT) was recorded for each sample to reflect the level of mRNA expression. A validation experiment in a given sample at different RNA concentrations confirmed the linear dependence of the CT value on the concentrations of *MUC5AC* and *GAPDH* and the consistency of ΔCT. ΔΔCT was used for relative mRNA quantification. The following primers were used for TaqMan gene expression assays (Life Technologies, Thermo Fisher Scientific): *MUC5AC* (HS01365601_m1) and *GAPDH* (4326317E).

2.6 | Immunofluorescence

The cells were grown on glass coverslips. After washing in PBS, the cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, blocked with PBS containing 1% BSA for 30 min, stained with a primary antibody, TOM20 (rabbit monoclonal antibody; 1:1000, Santa Cruz Biotechnology, sc-11415), and MUC5AC (Clone 45M1, Mouse monoclonal antibody; 1:50, Thermo Fisher Scientific, MS-145) for 60 min and stained with a secondary antibody against rabbit (Alexa fluor 488; 1:400) and mouse (Alexa fluor 488; 1:400) for 60 min. The cells were washed with PBS after each treatment. Coverslips were mounted with the Prolong Gold anti-fade reagent containing DAPI (Life Technologies, P36931). The cells were imaged using DeltaVision microscope (CORNES Technologies).

2.7 | Mitochondrial DNA extraction

Digitonin extracts from NCI-H292 cells were generated as previously described.[17–19] NCI-H292 cells were exposed to 100 μM H_2O_2 for an appropriate time, and the cells were divided into two equal aliquots. One aliquot was resuspended in 500 μL of 50 mM NaOH and boiled for 30 min to solubilize DNA. To neutralize the pH, 50 μL of 1 M Tris–HCl pH 8 was added; this served as a normalization control for total mtDNA. The second aliquot was resuspended in approximately 500 μL of buffer containing 150 mM NaCl, 50 mM HEPES pH 7.4, and 25 μg/mL digitonin (Fujifilm Wako, 043-21371). The homogenates were incubated for 10 min to allow selective plasma membrane permeabilization and then centrifuged at 980 ***g*** for 3 min three times to pellet intact cells.

Cytosolic supernatants were transferred to fresh tubes and spun at 17,000 ***g*** for 10 min to pellet any remaining cellular debris, yielding cytosolic preparations free of nuclear, mitochondrial, and endoplasmic reticulum contamination.

DNA was then isolated from pure cytosolic fractions and the control using classical phenol, phenol-chloroform, and chloroform extraction. DNA was extracted from both after RNase and proteinase K treatments. mtDNA was quantified by qPCR using the KAPA SYBR FAST qPCR Master Mix (Roche kk4605) on 1 μL of whole-cell extracts and cytosolic fractions with mtDNA primers (NAD1).[20] The thermal cycles were reported previously.[13,20] The CT values obtained for mtDNA and nuclear DNA abundance for whole-cell extracts served as normalization controls for each DNA value obtained from the cytosolic fractions. The following primers were used for SYBR Green assays: NAD1: FW 5′-ATACCCAT GGCCAACCTCCT-3′, RV 5′-GGGCCTTTGCGTAG TTGTAT-3′.

2.8 | cGAS and STING knockout using clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated system (Cas9)

To investigate the function of cGAS or STING molecules in NCI-H292 cells, we generated cGAS- or STING knockout (KO) cells using the CRISPR/Cas9 system. To utilize CRISPR/Cas9, we obtained plasmids to knock out cGAS or STING from a laboratory in the Netherlands.[21] FuGENE® HD Transfection Reagent (Promega E2311) was used for transfections (1 μg DNA/3 μL FuGENE) according to the manufacturer's protocol. After 24 h of transfection, a medium supplemented with 3.0 μg/mL puromycin (Sigma-Aldrich, P9620) was added for selection. After 72 h, the cells that survived selection were incubated. Single-cell clones were generated from these cell pools by limiting dilution, and DNA was isolated and tested for cGAS and STING gene integrity using PCR.

2.9 | PCR amplification to confirm integrity of cGAS and STING genes

DNA was isolated from single-cell clones seeded into 25 cm^2 dishes using classical phenol, phenol-chloroform, and chloroform extraction. DNA amplification was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems, 4398881) in a final volume of 20 μL containing 0.25 μM primer and 500 ng DNA template. The final reaction volume was 20 μL, using sterile double-distilled water. Vertii® 96-well thermal cycler (Applied Biosystems) was used for DNA amplification. The PCR conditions were as follows: 10 min at 95°C; 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and 72°C for 7 min. The PCR products were analyzed by electrophoresis on 2% agarose gel. Primers to confirm STING KO were: FW 5′-GAAGTGCCCAGCCAGAGC-3′, RV 3′-CACAGTCCTCC AGTAGCTGC-5′. Primers to confirm cGAS KO were: FW 5′-CGCCAGTAGTGCTTGGTTTC-3′, RV 3′-GAGGTCTT GGCTTCGTGGAG-5′.

2.10 | Western blotting

The cells were washed with PBS and scraped using a cell scraper. The cells were transferred to 1.5 mL microtubes and centrifuged at 5000 ***g*** for 3 min. Cells were lysed in 100 μL of lysis buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, 0.0125% bromothymol blue) and heated at 95°C for 5 min. Whole cell extracts were subjected to electrophoresis on 4%–20% Mini PROTEAN TGX gels (Bio-Rad, 4561095) and then transferred to Trans-Blot Turbo Transfer Pack membranes (0.2 μM PVDF, BIO-RAD, 1704156). Membranes were blocked with 5% BSA in Tris-Buffered Saline-Tween, TBST 0.001% azide for 60 min and probed with primary anti-STING (D2P2F) rabbit mAb (#13647, Cell Signaling Technology), anti-cGAS (D1D3G) rabbit mAb (#15102, Cell Signaling Technology), anti-EGF-Receptor (D38B1) XP rabbit mAb (#4267), and anti-β-actin (13E5) rabbit mAb (#4970, Cell Signaling Technology). The membranes were then washed with TBST and incubated with a secondary donkey anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (HRP) (Abcam, ab205722) for 1 h. Proteins were detected using a chemiluminescence detection system (ECL Prime Western Blot Detection System; GE Healthcare, RPN2232) according to the manufacturer's instructions.

2.11 | Statistical analysis

For comparisons of multiple time points to the 0 h control, and that of different concentrations of H_2O_2 to unstimulated controls, statistical significance was determined using Dunnett's test, as implemented in IBM SPSS statistics 24 (IBM Corp. Released 2016, IBM SPSS Statistics for Windows, Version 24.0; IBM Corp.).

Other multiple comparisons were performed using the Tukey's test. Single comparisons were performed using the Mann–Whitney test. The significance was set at $p \leq 0.05$.

3 | RESULTS

3.1 | H_2O_2 induces *MUC5AC* transcriptional activity

The expression levels of *MUC5AC* mRNA in NHBE cells after H_2O_2 stimulation were determined by quantitative reverse transcription polymerase chain reaction, which were found to increase significantly after 24 h of stimulation (Figure 1A). Further, similar to NHBE cells, stimulation of NCI-H292 cells with H_2O_2 for 24 h increased the *MUC5AC* mRNA expression significantly (Figure 1B). Therefore, we studied the detailed pathways using NCI-H292 cells. No difference was observed in the cell viability evaluated after stimulation with 100 μM H_2O_2 up to 48 h post-stimulation than that at baseline (Figure 1C). Furthermore, a significant amount of *MUC5AC* mRNA levels peaked after 24 h of stimulation (Figure 1D). Similar to H_2O_2, CSE, which causes oxidative stress, also showed a significant increase in *MUC5AC* transcriptional activity (Figure 1E).

3.2 | H_2O_2 affects mitochondrial organization causing mtDNA release into cytoplasm

Mitochondria have been reported to change their form under stress.[22] Therefore, we examined whether oxidative stress induced by H_2O_2 caused morphological changes in the mitochondria. Using immunostaining, we found that the mitochondria were distributed throughout the cytoplasm before stimulation, but were densely packed after stimulation (Figure 2A). Furthermore, mitochondria are shown to release mtDNA into the cytoplasm in response to stress. To determine whether mtDNA was released into the cytoplasm, quantification of the amount revealed a significant increase at 1 and 6 h after H_2O_2 stimulation (Figure 2B).

3.3 | Mitochondrial damage mediates H_2O_2-induced *MUC5AC* expression

We investigated the involvement of mitochondrial damage in H_2O_2-induced increase in *MUC5AC* expression. Toward this, we assessed whether the protection of mitochondria from oxidative stress blocked *MUC5AC* mRNA expression. MitoTEMPO, an antioxidant that targets and protects mitochondria, reduced H_2O_2-induced *MUC5AC* mRNA expression and protein significantly as compared to that in untreated controls (Figure 2C; Figure S1A,B). Further, we analyzed *MUC5AC* mRNA expression after treatment with thapsigargin, an endoplasmic reticulum stressor, that triggers the release of mtDNA into the cytoplasm.[13] Thapsigargin induced a significant increase in *MUC5AC* mRNA expression (Figure 2D). These results indicate that mitochondrial damage induces *MUC5AC* expression and its protection from oxidative stress and inhibition of mtDNA release into the cytoplasm inhibits *MUC5AC* expression.

3.4 | H_2O_2 induces MUC5AC expression via STING, and not via cGAS

Our results suggest that cytoplasmic mtDNA is involved in the *MUC5AC* mRNA expression. Toward this, we transfected poly(dA:dT) into the cytoplasm as an exogenous cytoplasmic DNA and observed a significant induction of *MUC5AC* mRNA expression (Figure 3A). Further, the cGAS-STING pathway is known to be involved in the immune response to intracytoplasmic DNA.[23] Therefore, we generated STING KO cells using the CRISPR-Cas9 system in NCI-H292 cells. STING KO was confirmed to be devoid of STING at both the genomic DNA and protein levels (Figure S2A,B). *MUC5AC* transcriptional activity increased by cytosolic introduction of poly(dA:dT) was significantly suppressed in STING KO cells (Figure 3B). Stimulation with cGAMP, an upstream molecule of STING, was shown to induce *MUC5AC* mRNA expression in wild-type cells but not in STING KO cells (Figure S2C). These results indicated that *MUC5AC* mRNA expression induced by cytoplasmic DNA is dependent on STING.

Next, we investigated whether STING was involved in H_2O_2-induced *MUC5AC* expression. When stimulated with H_2O_2, *MUC5AC* mRNA and protein increased in a concentration-dependent manner in WT cells, but were suppressed in STING KO cells (Figure 3C; Figure S1C,D). However, unlike H_2O_2, CSE induced *MUC5AC* mRNA expression in both STING KO and WT cells (Figure 3D). Next, we used TGF-α, a ligand of the EGFR, which is a well-known inducer of *MUC5AC*.[24,25] The EGFR-MAPK pathway has been previously reported to be involved in *MUC5AC* transcriptional activity in H_2O_2 treatment.[25] Therefore, we tested the expression and function of EGFR in STING KO cells. EGFR expression was found to be comparable in STING KO and WT (Figure 3E). Further, TGF-α induced a significant *MUC5AC* expression in both (Figure 3F), thereby suggesting that an equivalent EGFR expression in STING KO as well as in WT cells had a specific role in *MUC5AC* expression. Subsequently, we confirmed the cGAS KO cells for cGAS expression (Figure S2D,E). Interestingly, unlike STING KO cells, *MUC5AC* transcription was not repressed in cGAS KO cells when compared to that in WT cells (Figure 3G).

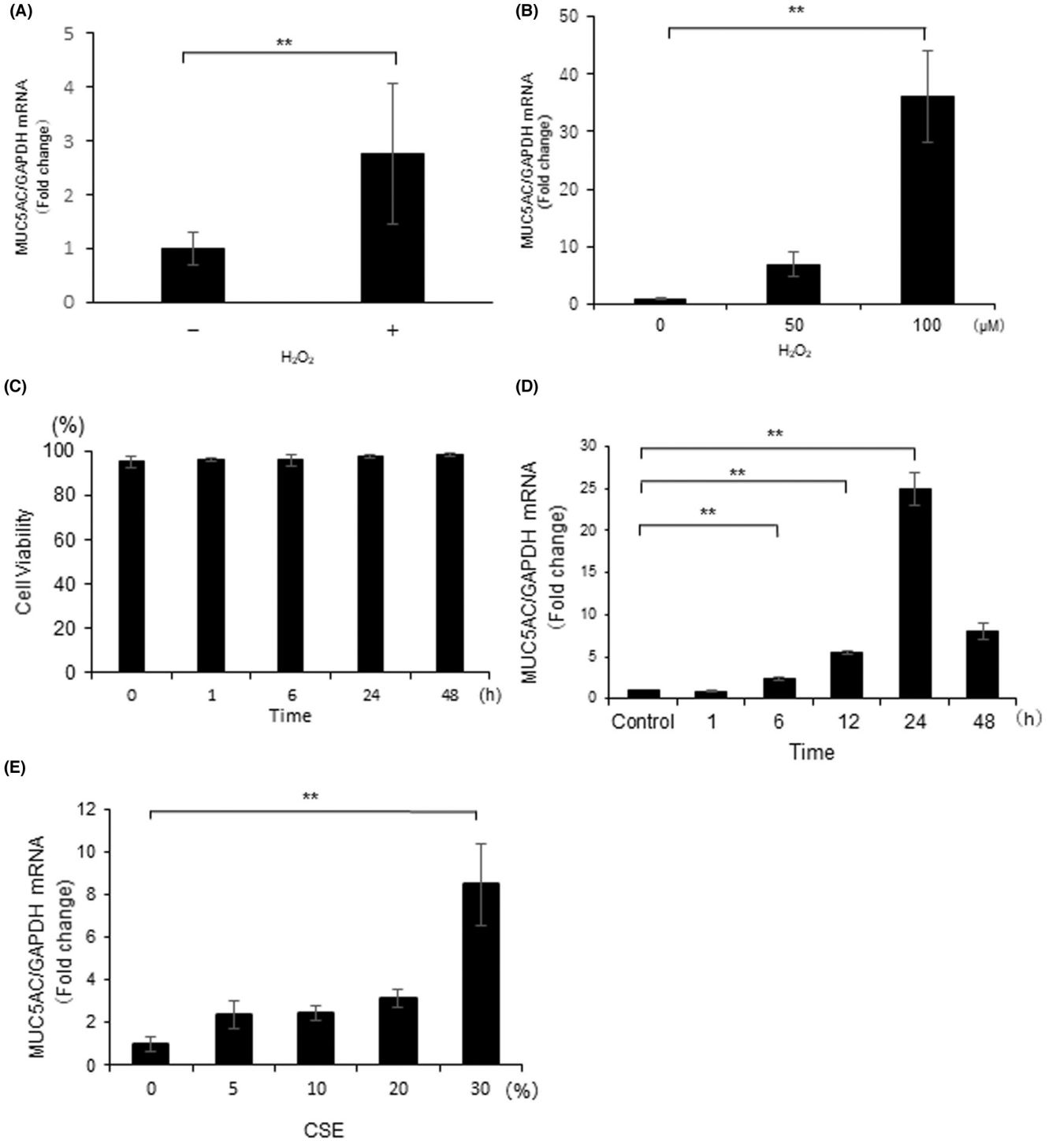

FIGURE 1 *MUC5AC* mRNA expression induced by oxidative stress. (A) *MUC5AC* mRNA levels in NHBE cells stimulated with 500 μM H_2O_2 for 24 h ($n = 8$). (B) *MUC5AC* mRNA levels in NCI-H292 cells stimulated with 0–100 μM H_2O_2 for 24 h ($n = 4$). (C) Viability of NCI-H292 cells treated with 100 μM H_2O_2 for 0–48 h ($n = 4$). (D) *MUC5AC* mRNA levels in NCI-H292 cells stimulated with 100 μM H_2O_2 for 1–48 h ($n = 4$). (E) *MUC5AC* mRNA expression in NCI-H292 cells stimulated with 0%–30% CSE for 24 h ($n = 4$). The results are presented as mean ± standard deviation (SD). Significant differences are indicated as $^{**}p < 0.01$. Significance was calculated using the Mann–Whitney test (A), Dunnett's test (B, C, E), and Tukey's test (D).

Finally, we investigated whether mitochondrial damage and STING activation are involved in oxidative stress-induced *MUC5AC* mRNA expression in NHBE cells as well as in NCI-H292 cells. In NHBE cells, *MUC5AC* transcriptional activity in response to H_2O_2 stimulation was significantly suppressed by pretreatment with MitoTEMPO and the STING inhibitor H-151 (Figure 4).

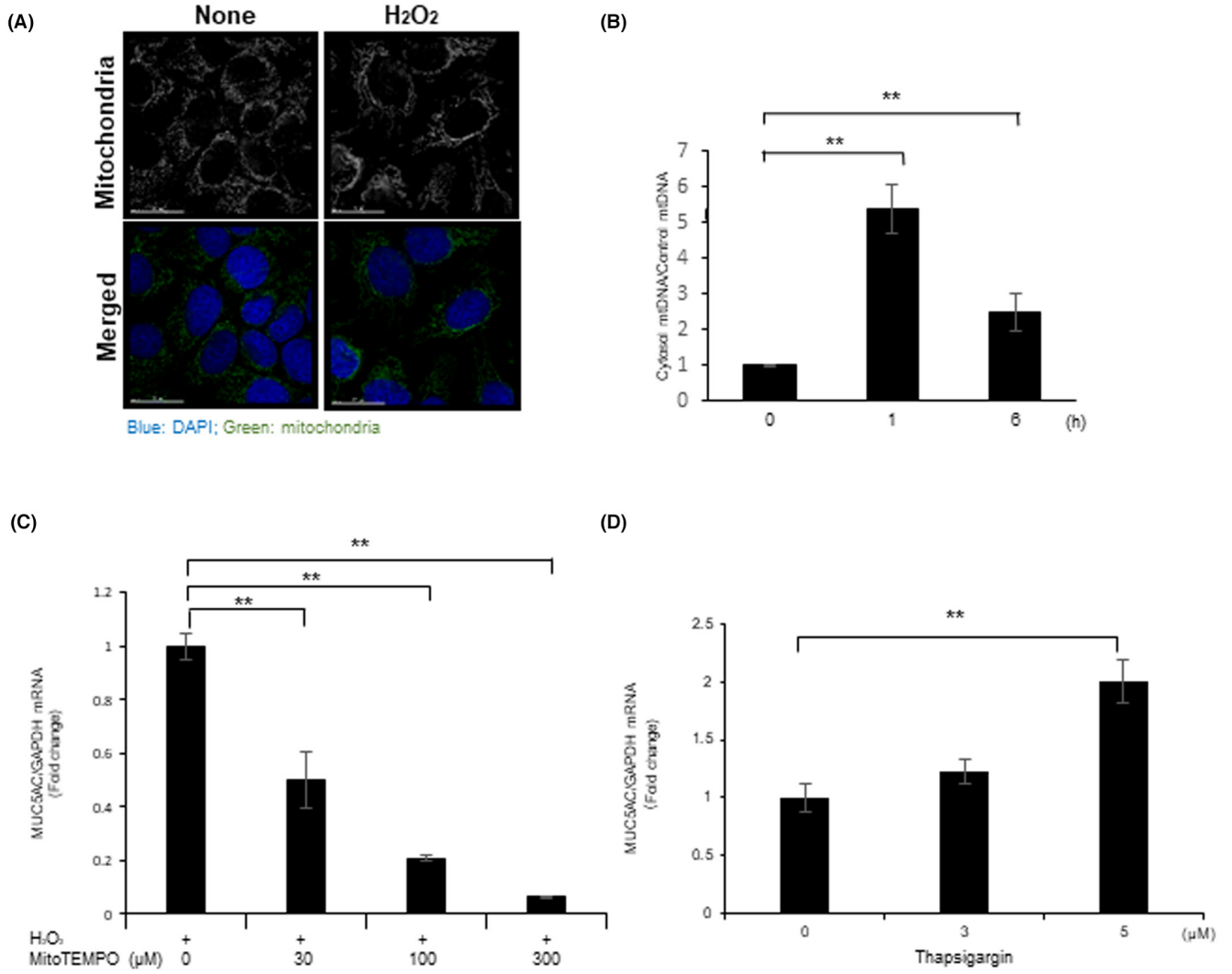

FIGURE 2 Mitochondrial DNA released into the cytoplasm by H_2O_2 stimulation is involved in *MUC5AC* transcription induction. (A) Images of immunofluorescence staining of mitochondria of NCI-H292 with anti-TOM20 antibody when stimulated with H_2O_2 for 24 h. (left) No stimulation, (right) H_2O_2 100 μM. Green: mitochondria (anti-TOM20 antibody); Blue: DAPI. Original magnification, 400×; bar = 15 μm. (B) The amount of mtDNA (NAD1) in the cytoplasm at 1 and 6 h after 100 μM H_2O_2 stimulation ($n = 4$). (C) *MUC5AC* mRNA in NCI-H292 cells pretreated with MitoTEMPO (0, 30, 100, and 300 μM) and stimulated with 100 μM H_2O_2 for 24 h ($n = 4$). (D) *MUC5AC* mRNA in NCI-H292 cells treated with 0–5 μM Thapsigargin for 4 h ($n = 4$). The results are represented as mean ± SD. Significant differences are indicated by $^{**}p < 0.01$. Significance was calculated using the Dunnett's test (B–D).

4 | DISCUSSION

Mitochondria are the main source of endogenous ROS in cells but are also the primary target of exogenous ROS.[5,26] Mitochondria are known to change their morphology in response to the degree of oxidative stress, and this has been reported in lung tissue from COPD patients and in HBECs exposed to CSE.[27–29] In addition, mitochondria are involved in innate immunity as damage-associated molecular patterns, which rapidly release mitochondrial molecules such as mtDNA under stress, causing inflammation.[20,30,31] mtDNA is more susceptible to oxidative damage than nuclear DNA[5] because mtDNA does not contain histones that play a protective role against oxidative stress, and the repair function of mtDNA is low.[32] In the present study, stimulation of NCI-H292 cells with 100 μM H_2O_2 resulted in changes in mitochondrial morphology, with an increase in cytoplasmic mtDNA 1 h after H_2O_2 stimulation. These changes may represent a rapid response of the mitochondria as innate immunity to mild oxidative stress that does not cause cell death. Furthermore, in the present study, pre-administration of the mitochondria-targeted antioxidant MitoTEMPO significantly suppressed H_2O_2-stimulated transcriptional activity of *MUC5AC*. No report has shown a direct relationship between mitochondrial oxidative stress and transcriptional induction of *MUC5AC* so far. This is

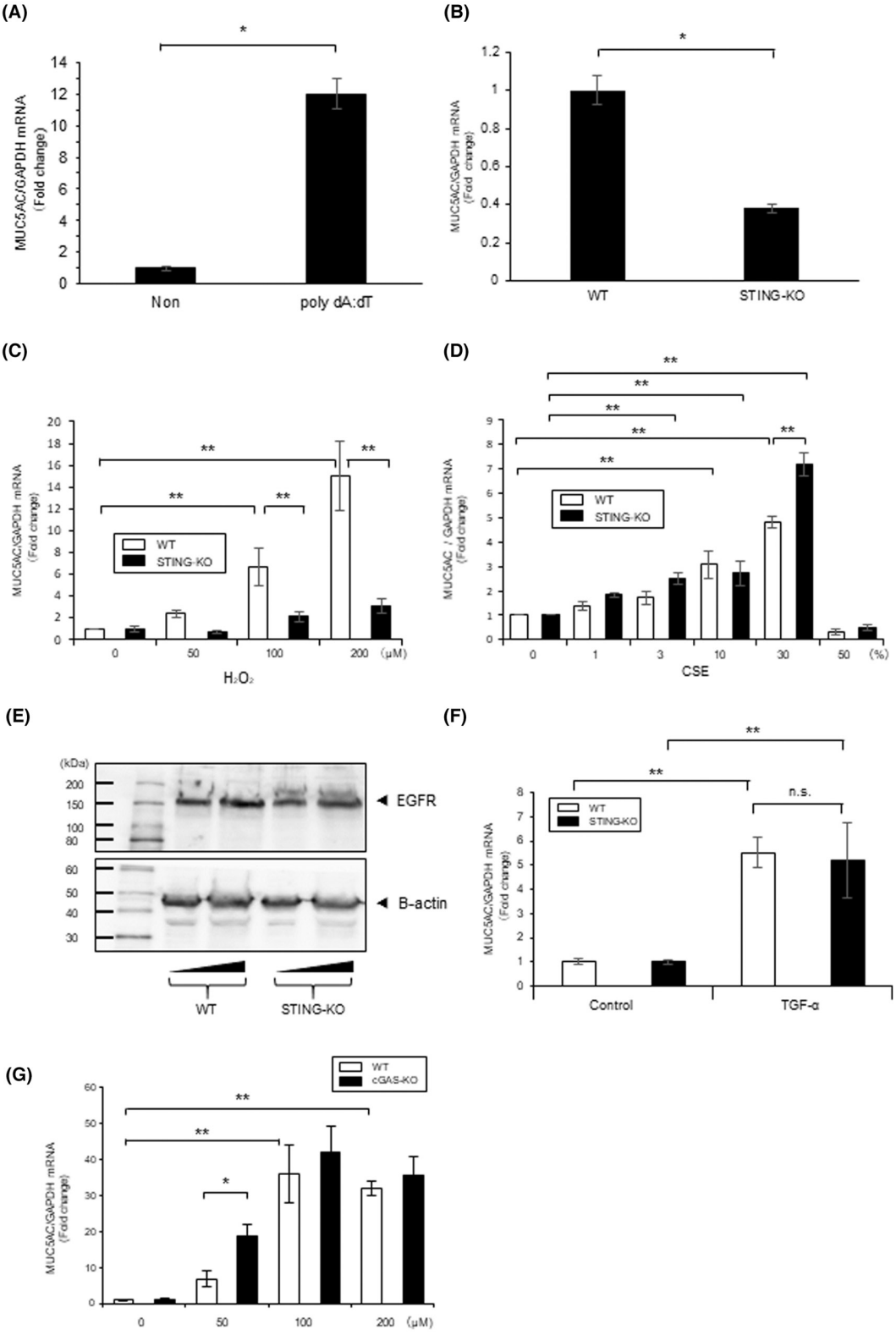

FIGURE 3 STING is involved in H_2O_2-stimulated *MUC5AC* transcriptional activity. (A) *MUC5AC* mRNA expression in NCI-H292 cells induced after transfection with poly(dA:dT) ($n = 4$). (B) *MUC5AC* mRNA in wild-type (WT) and STING KO NCI-H292 cells induced by poly(dA:dT) ($n = 4$). (C, D) *MUC5AC* mRNA in WT and STING KO NCI-H292 cells induced by (C) H_2O_2 ($n = 4$) and (D) CSE ($n = 4$) for 24 h. (E) Epidermal growth factor receptor protein expression in WT and STING KO NCI-H292 cells. (F) *MUC5AC* mRNA in WT and STING KO NCI-H292 cells induced by transforming growth factor-alpha for 6 h ($n = 4$). (G) *MUC5AC* mRNA in WT and cGAS KO NCI-H292 cells induced by H_2O_2 for 24 h ($n = 4$). The results are represented as mean ± SD. Significant differences are indicated by $^*p < 0.05$, $^{**}p < 0.01$ and ns, not significant. Significance was calculated using the Mann–Whitney test (A, B) and Tukey's test (C, D, F, G).

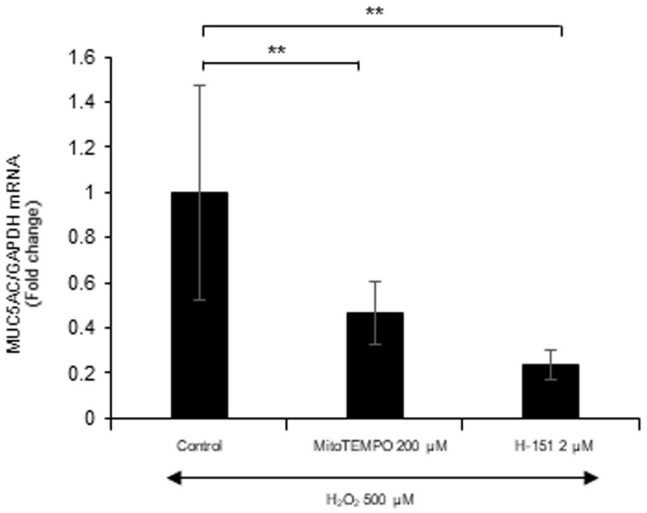

FIGURE 4 Mitochondrial damage and STING are involved in H_2O_2-stimulated *MUC5AC* transcriptional activity in NHBE cells. *MUC5AC* mRNA expression upon H_2O_2 stimulation in NHBE cells pretreated with 200 μM MitoTEMPO and 2 μM STING inhibitor H-151 ($n = 8$). Significant differences are indicated by $p < 0.01$. Significance was calculated using the Dunnett's test.

an important finding that indicates the involvement of mitochondria in airway mucin production.

Involvement of the EGFR-MAPK pathway in the production of *MUC5AC* by H_2O_2-induced oxidative stress is known[25]; however, the involvement of STING has not been described. We are the first to report the role of STING toward *MUC5AC* transcriptional activation. STING is activated by a variety of DNAs in the cytoplasm, including viruses and bacteria, as well as by mtDNA.[8] Although cGAS is a representative upstream molecule of STING, there are other cytoplasmic DNA sensors that activate STING. In the present study, *MUC5AC* mRNA levels were not decreased in cGAS KO cells, suggesting that STING may be activated by DNA sensors other than cGAS.[33,34] Moreover, the relationship between the EGFR-MAPK and STING pathways and H_2O_2-stimulated *MUC5AC* transcription has not been reported. In this study, *MUC5AC* transcription was induced in both STING KO and wild-type cells by stimulation with TGF-α, a ligand for EGFR. We speculate that STING may be involved in the induction of *MUC5AC* transcription by oxidative stress, either upstream of EGFR or in a pathway different from EGFR. Furthermore, it has been reported that oxidation of STING by ROS suppresses the type I interferon response during viral infection.[35] We observed that stimulation of NCI-H292 cells with H_2O_2 reduced the transcriptional activity of interferon-β (M. Ota, Y. Nishida, T. Takizawa, unpublished data). The downstream pathways of STING were not fully investigated, but the transcriptional activity of MUC5AC is likely induced by pathways different from type I interferons. Further studies are needed to elucidate the detailed signaling pathways involved.

Cigarette smoke extract increases *MUC5AC* transcriptional activity in a STING-independent manner. Cigarette smoke contains a large number of oxidants and over 4000 chemicals, which may stimulate pathways other than STING to increase *MUC5AC* mRNA expression.[36,37] For example, EGFR is involved in a CSE-induced increase of MUC5AC mRNA.[38] Besides, JNK and AP-1 pathways have also been implicated in CSE-induced MUC5AC expression.[39] These data suggest multiple pathways in MUC5AC transcriptional activation by cigarette smoke. However, cigarette smoke is known to cause mitochondrial damage.[40] The current results do not necessarily rule out the involvement of STING-mediated pathways in the oxidative stress-induced mucin production due to cigarette smoke.

A limitation of this study is that most findings were obtained with a single cell line, that is, NCI-H292 cells. We used NHBE cells to reproduce a part of, but not all, the findings in conditions closer to physiology. Although NCI-H292 cells have been widely used for airway mucin studies, the findings based on a single cell line may not be applicable widely.

Thus, in this study, we found that the oxidative stress induced by H_2O_2 caused mitochondrial morphological changes and the release of mtDNA into the cytoplasm. H_2O_2 stimulation also upregulated *MUC5AC* transcription, which is ameliorated by attenuation of mitochondrial damage. Furthermore, STING was required for oxidative stress-induced *MUC5AC* expression. These results support our hypothesis that mitochondrial damage caused by oxidative stress releases mtDNA into the cytoplasm, activates the STING-mediated pathway, and eventually induces *MUC5AC* transcriptional activation.

Our results indicate that the innate immune mechanism is strongly involved in airway mucin production induced by oxidative stress. Furthermore, mitochondria play an important role in the initial response of the airway epithelium to oxidative stress. Thus, mitochondria and STING are possible targets for the treatment of diseases associated with oxidative stress, such as asthma and COPD.

AUTHOR CONTRIBUTIONS

Y. Nishida, H. Arakawa, and T. Takizawa conceived and designed the research; M. Ota and N. Arakawa performed the research and acquired the data. H. Yagi, A. Tanaka, K. Sato, T. Inoue, S. Yamada, T. Ishige, and Y. Kobayashi analyzed and interpreted the data. All authors were involved in drafting and revising the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or Supporting Information of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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